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Characterization of culturable bacterial communities associated with cage cultured fish under different stocking densities in Setiu Wetlands, Terengganu, Malaysia.

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Characterization of culturable bacterial communities associated with cage cultured fish under different stocking densities in Setiu Wetlands, Terengganu, Malaysia

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Abstract

Objective: The study objective was to evaluate the effects of fish stocking density on the prevalence of pathogens isolated from sixty clinically healthy fishes reared under different densities in floating net-cages in Setiu lagoon, Terengganu state, Malaysia.

Results: The water temperature, pH, dissolved oxygen, total dissolved solids, salinity, water clarity, depth, and coliform concentration, were all determined within the fish cages. The healthy fish samples were randomly collected from 3 sites (A, B, and C) in cages with low and high stocking densities at each site. The bacteria were isolated from the skin, gills, kidneys, and liver of each fish sample, followed by identification to species level using the VITEK-2 system. Water quality parameters showed no differences between the cages of low and high fish densities except for site C.

The total number of isolates, microbial species, and the number of pathogens isolated from fish revealed no significant difference between the fish stocked in low and high densities. A total of 25 bacterial species were isolated, which included 14 gram-positive and 11 gram-negative.

Keywords: Cage aquaculture, fish stocking density, pathogens, water quality, coliforms.

Introduction

Nowadays, the demand for food is increasing especially for fish consumption. Therefore, solutions have been introduced mainly by intensive aquaculture systems that have become the point of economic interest of many farmers. In Setiu Wetlands, Malaysia, this area has a significant potential for both aquaculture activities and fisheries such as floating net cages that have become one of the fastest flourishing economic activities. Stocking large quantities of biomass/fish numbers with larger feed inputs in the fish ponds allows more financial income, but it can eventually lead to disease outbreaks [7], [8]. Other studies have indicated that intensive stocking fish densities in cage cultures increase the competition for food, living space, and social interactions between the fish individuals (aggressive behaviors) [23]. Moreover, it causes lowering of individual fish growth and increasing cortisol (stress hormone) levels [25, 27]. In addition, fish compression at the bed of the cage culture due to gravitational fish weight, which can cause abrasions and injuries by spines of the adjacent fish [6].

The intensive production of fish in cages might induce deleterious effects on the ambient aquatic environment such as water pollution resulting from excess of uneaten fish feed and fish excreta from cages to the surrounding environment. makes the cage farms as an extra source of nutrients that encourage microbial growth. thus, poor water quality is considered a source of stress that reduces the health of cultured fish and makes them more susceptible to bacterial infections [29].

This study was undertaken to evaluate the effects of fish stocking density reared in commercial cage cultures associated with prevalence of pathogens and its water quality parameters.

Main text

Materials & Methods

Study area

The study was conducted at a private commercial fish farm located in Setiu Wetland, along the north coast of the Terengganu state, Malaysia (Additional Figure S1 (A) & (B)).

Culture cages

The fish farm has a total of 50 floating net-cages filled with the tiger grouper (*Epinephelus fuscoguttatus*) and the Asian seabass (*Lates calcarifer*). These cages came in two different depths (1.7 m in sites A and C, while 1.9 m in site B). The fish stocking density was determined by the number of fish per cage of the three-dimensional medium [6] (Table 1).

Fish Sampling

The fish samplings were conducted in July 2018 during the dry season from three sites A, B, and C (Figure S1). Sixty fish (700 – 1000 gm) were randomly collected from 3 sites (Table 1). According to the farm history, there were no disease outbreaks on this farm.

Water quality measurements

Temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS), salinity and water transparency (WT) were determined using multiprobe sensor (YSI, 556 MPS, USA) and Secchi disk (Wildlife Supply Company, USA) within the cages before, during and after the fish sampling; below the water surface, 1 m, and 2 m . The water flow rate and depth of the water column were assessed using a current water meter (Valeport, UK), and ultrasonic depth sensor (Speed Tech SM-5, USA), respectively. Geographic Positioning System (GPS) coordinates were taken using a Garmin GPS (SNAP 276C, US). The study area map was created using: Google Earth V. 7.3 and ArcMap V. 10.3.

Microbial water quality

Water samples were collected in sterile plastic containers of 50 ml within the fish sampling cages 15-20 cm below the water surface. The fecal contamination in the water samples was analysed according to the guidelines [3], [12], [17], [28].

Bacterial isolation methods

The selected fish was observed for any signs of lesions. Necropsy was done according to [18].

The bacteria were isolated in three replicates from the skin, gills, liver and kidneys, plated on Zobell Marine Agar (HIMEDIA, India), Thiosulphate-Citrate Bile Salts Sucrose agar (Difco™, USA), ready to use Blood Agar plates (Thermo Scientific Microbiology Sdn Bhd, Malaysia) and incubated at 37°C for 24 hours according to

[22]. The purified isolates were identified [5], [7], [9], [15], [21] to species level using an automated bacterial identification instrument (VITEK-2, BioMérieux, USA).

Data analysis

The data analysis was performed using R software version 3.3.2, packages used were: psych, and stats. The assumptions of parametric analysis were not met. Non-parametric tests were done according to [13], [16].

The Spearman's rank correlation test (ρ) was used to determine the correlations between the water quality parameters, fish density and the total number of microbial isolates.

All significant differences were $p < 0.001$ (with Bonferroni correction) unless mentioned otherwise.

Results

Physicochemical parameters

Mean values of the water parameters with their standard deviations ($\pm$ SD) are presented in Table S1.

All water quality parameters were not significantly different between the cages (A1 and A2), and (B1 and B2), with the exception for the pH in both B sites and water flow rate between all sampling sites ($P < 0.05$).

The sites (C1 and C2) showed a significant difference ($P < 0.05$) in all water parameters except for DO and Secchi depth readings.

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4 There was a significant difference in the observed values of water samples
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6 between the sampling sites (A, B and C) for each parameter ($P < 0.05$), except for the
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8 WT.
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10 11 12 13 14 **Microbiological parameters**

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16 Total coliform (TCF) counts, fecal coliforms counts and thermotolerant fecal
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18 coliforms (FC) are shown in Tables S2, S3, and S4.
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21 22 23 **Bacterial isolates**

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25 A total of 88 bacterial strains were isolated from 60 visually healthy fish except for
26
27 one sample that consisted of congested and swollen kidneys upon necropsy,
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30 The number of Alpha-hemolysin (α) and Beta-hemolysin (β) bacteria was higher
31
32 than the Gamma strains number (γ).
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36 The number of identified species was 25, which were 56% gram-positive bacteria
37
38 and 44% gram-negative bacteria. The dominant bacteria were mostly gram-positive
39
40 (69.32%) compared to gram-negative ones (28.41%), (Tables S5, S6) (Figure 2,
41
42 Figure S2).
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45 46 47 48 **Correlation parameters**

49
50 Negative strong and significant correlations were observed for: salinity, TDS,
51
52 Secchi depth and pH with the total number of bacterial isolates ($P < 0.05$) (Table S7,
53
54 Figure S3). Temperature, water flow, TCF, FC concentrations and fish density were
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56 non-significant ($P > 0.05$). The DO revealed a non-significant negative moderate
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correlation ($P > 0.05$). Fish density showed a non-significant and moderate positive correlation with the total bacterial counts ($P > 0.05$).

Discussion & Conclusions

The Physicochemical parameters results indicated water temperature fluctuations within the sampling sites with the lowest temperature at site B due to the highest freshwater discharge from the estuary towards the cages, which frequently carried the metabolic wastes of the fish away from the cages. This variation was attributed to the time of day of sampling, and on the tide [24].

Site C2 showed the highest temperature and lowest salinity, TDS, and pH compared with C1. This site had the highest fish density with a potentially high presence of fish feed that could lead to a high respiration rate due to the fish metabolic needs linked to the digestion of food, and increase fish excreta plus the effect of low water currents at site C1 and site C2. This led to increased temperature and lowered pH, besides the low water flow that was probably insufficient to wash away the metabolic wastes. The excess fish food and feces discharged from aquaculture are a major factor contributing to the increased organic decomposition rate, which is responsible for decreasing DO and pH in the water column [23, 24].

Noteworthy, both sites A2 and C1 received the lowest water inflow, A2 showed significantly higher mean DO, salinity, TDS and pH ($P < 0.05$), except for the temperature. This could be due to widely buffered condition of site A from the seawater currents during high tide, conversely to site C that was subjected more to freshwater fluxes from the estuary, which was markedly discerned with the decreased salinity, TDS, DO, pH and WT at site C; different oxygen requirement of different fish species.

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4 A study reported the DO concentration at the same Wetland area was low due to
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7 the respiration activities of the fish inside the cage area [24].
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10 The salinity had exceeded the optimal range (15-25) ppt for the grouper at site A
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12 [20] and pH levels were less than the optimal range (7.5 - 8.5) for the seabass [2] at
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14 site C2, (Table S1), which means the grouper fish in this study were under salinity
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16 stress, and the seabass species at site C2 exposed to non-optimal pH.
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20 Although the physicochemical water parameters were not different between the
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22 cages of low and high fish densities, The salinity, TDS, pH, and WT that were
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24 observed to have strong negative associations with the presence of total bacterial
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26 isolations in all the sampling sites.
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30 High TCF at site C1, and the lowest at site A, while FC were also the lowest at site
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32 A, which may be due to the direct discharge of wastewater from septic tank, industrial
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34 wastewater to the Setiu river and due to the vicinity of C sites from the river mouth.
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36 Contrary to A sites that are mostly washed by sea water currents during the high tides.
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40 The low water flow rate and high-water clarity at site A likely facilitated the
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42 penetration of solar light into the water column, in addition to the high salinity and pH
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44 during solar exposure were considered as exogenous factors that play an important
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46 role in accelerated microbial decay rate [19]. Besides, polluted river water could have
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48 brought coliforms from the surrounding residential area to the estuary. Hence, the
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50 variation of coliform concentrations at sites B and C are associated with
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52 hydrodynamics driven by water flow, salinity changes, and the sedimentation rate of
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54 the suspended or dissolved particles.
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4 The correlation coefficient between fish density and the total number of microbial
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6 isolates imply that fish density has no direct effect on the total number of bacterial
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8 isolates. A study postulated that 50 fish/m³ is optimum stocking density while 75 to
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10 100 fish/m³ is considered high to Nile tilapia species because within those densities a
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12 rise in plasma cortisol concentrations was evident due to social stress between fish
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14 individuals as well as the survival rate was reduced by 60% [4]. Researchers
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16 recommended the optimum density should not exceed 35 to 43 fish/m³ [10]. This
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18 suggests that our selected fish stocking densities were within normal ranges in this
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20 study.
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26 Similarly, other studies have reported that physico-chemical parameters showed
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28 their importance in influencing the occurrence and the density of bacterial populations
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30 [1], [11], [14].
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33 The significant difference in total pathogens isolation (Figure 1) between the sites
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35 A1 and A2 with the site C2, is attributed to the differences in the fish species and
36
37 water quality.
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40 Generally, the salinity, TDS, pH, and WT were strongly negatively correlated with
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42 the occurrence of total bacterial isolates while neither the fish density nor the coliform
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44 concentrations did show a significant correlation with total bacterial isolates among all
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46 sites.
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50 The water characteristics have favored the cultured seabass to harbor more
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52 pathogens at sites B and C, such pathogens may be also the result of the diseased or
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54 carrier fish. A non-optimal pH level was noticed at site C2 with a potential of high
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56 microbial load surrounding the cages. The non-optimal salinity levels are considered a
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58 stressful condition to grouper species [20], which led to some bacterial infections.
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4 Our findings in the present study indicated that fish density has no effects on
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6 the pathogen numbers, species numbers, and overall bacterial isolations. This
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8 suggests that the fish stocking densities were within the optimum in this study.
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12 In this survey, we isolated several bacterial species from the fish, which may be
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14 associated with the nature of the environment and water quality surrounding the
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16 cages. However, the environmental variables are far more easily exposed to
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18 fluctuations, which makes it necessary for further study to investigate such
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20 relationships during the wet season.
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27 28 **Limitations** 29

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31 • A comprehensive estimation of the health risk of this farm is required by using
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33 advanced molecular techniques (e.g., examine the virulence genes of
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35 pathogenic isolates) to address the problem of infected fish with no clinical
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37 signs.
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41 • In order to have a conclusive decision about the study results, it is necessary to
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43 have similar experiments in the wet season.
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List of abbreviations

DO: Dissolved Oxygen

FC: Thermotolerant fecal coliforms

GPS: Geographic Positioning System

SD: Standard deviation

TCF: Total coliform

TDS: Total Dissolved Solids

WT: Water Transparency

α : Alpha-hemolysin

β : Beta-hemolysin

γ : Gamma-hemolysin

Ethics declarations

Ethics approval and consent to participate

This study was approved by the Ethical Review Committee in Universiti Malaysia Terengganu (written consent).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [in supplementary files]

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Fieldwork, sample collection, all laboratory work, and data analysis were carried out by Mohammed M.Tawfeeq. Mohd Effendy, Musa Najiah, and Isabelle G. are equally contributed to the manuscript editing, pertinent support, and valuable comments. All authors contributed to the article and approved the submitted version.

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Table 1. Study sampling design, fish species, and their densities among the sampling sites.

Cages	Total fish no. stocked per net- cage	Stocking density (fish/m ³)	Sampled Fish	Fish species	Sampling sites	GPS coordinates
Low fish density	300	12	10	Grouper	*A1	N 05°40'59.6" E 102°42'40.7"
	400	14.32	10	Seabass	^a B1	N 05°40'54.5" E 102°42'42.2"
	600	24	10	Seabass	*C1	N 05°40'45.1" E 102°42'50.3"
High fish density	600	24	10	Grouper	*A2	N 05°40'59.5" E 102°42'40.7"
	1000	29.23	10	Seabass	^w B2	N 5°40'54.5" E 102°42'41.4"
	1000	32.68	10	Seabass	^b C2	N 05°40'45.6" E 102°42'50.0"
Total	3600	136.23	60			

The volume of submerged net-cages in the water: * = 24.99 m³, ^a = 27.93, ^b = 30.6 m³, ^w = 34.2 m³.

Figure 1. Total numbers of pathogenic bacteria isolated from fish stocked under low and high densities at the different sampling sites. (significance levels: a = 0.008, c = 0.03, ns = non-significant).

Figure 2. Total numbers of bacteria isolated from fish stock in high densities at the different sampling sites. (significance levels: a, c = 0.025, b=0.03, d= 0.059, ns = non-significant)

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